Bioinformatics of Alcoholic Brain Proteome Studies
6.1 Introduction

Genomic and proteomic analyses are essential for understanding the underlying factors involved in human disease. However, with the sheer amount of experimental data these high-throughput studies generate, specialized computational technologies are often required for data analysis (Phan et al., 2006). The use of analytical tools such as Ingenuity Pathway Analysis software (IPA; Ingenuity® Systems, USA; www.ingenuity.com) may not only help identify important interactions and associations within the dataset, but introduce objectivity into the interpretation of these high-throughput study results. Furthermore, by using such computing tools these identified disrupted proteins may be viewed as whole systems rather than fragmented pieces of a hypothetical puzzle.

In the preceding Chapters of this thesis, a large number of proteins were identified in the BA9 white and grey matter and cerebellar vermis of uncomplicated alcoholics and alcoholics diagnosed with liver cirrhosis. Some of these altered protein abundance profiles were shared between the studies, suggesting a disruption in common pathways, e.g. a disturbance in energy metabolism perhaps related to thiamine deficiency, which may be important for the pathophysiology of alcohol-related brain damage. Yet, some results were region specific or correlated to the presence of liver cirrhosis. Using programs such as IPA, further understanding of how these protein systems are disrupted in alcoholism may be achieved.
6.2 Methods

Pathway analyses of the alcohol studies were performed using IPA software. This software program calculates the probability that the genes associated with our dataset (identified proteins) are involved in particular biological processes and metabolic and signaling pathways. Furthermore, this program helps to determine which of these are most significantly disrupted in each study. No directionality is associated with these relationships, i.e. the function shouldn’t be interpreted as being increased or decreased. Significance values were calculated using the right-tailed Fisher’s Exact Test by comparing the number of proteins that occur in a given pathway relative to the total number of occurrences of those proteins in all functional annotations stored in the Ingenuity Pathways Knowledge Base.

Using IPA-Biomarker™, filters were applied to the datasets to identify and prioritize the most relevant and promising molecular biomarker candidates. The biomarker filter was set based on the following contextual information;

a) Genes expressed in humans,

b) Genes expressed in tissues restricted to the nervous system, and

c) Genes that are mechanistically connected to metabolic disease, neurological disease, nutritional disease, organ injury/abnormalities and/or psychological disorders.

Using these filters, biomarker candidates that discriminate between or are common to the different groups of alcoholics and brain regions were isolated.
6.3 Cellular and Disease Process Analyses

The following Figures (6.2.1-3) depict an array of biological functions that are significantly associated with the proteins identified in the BA9 white and grey matter studies and the cerebellar vermis study. Functional associations were very similar between the uncomplicated alcoholics and alcoholics with liver cirrhosis across all brain regions studied. This applies not to only the type of biological functions, but also the level of association. These associations included cellular assembly and organisation, cell morphology, cell-to-cell signaling and interaction, neurological disease and lipid metabolism. More disparity between the alcohol groups, however, is seen in the cerebellar vermis study. Here, cellular function and maintenance, small molecule biochemistry, protein trafficking, nucleic acid metabolism and protein folding associations were either more pronounced in alcoholics with liver cirrhosis or were associated with this alcohol group alone.
Figure 6.2.1: Biological functions significantly associated with the proteins identified in the BA9 white matter of human alcoholics. Figure was adapted from Ingenuity® Pathway Analysis software. Significance threshold set at $-\log(0.05)$. 
Figure 6.2.2: Biological functions significantly associated with the BA9 grey matter proteins identified in human alcoholics. Figure was adapted from Ingenuity® Pathway Analysis software. Significance threshold set at $-\log(0.05)$. 
Figure 6.2.3: Biological functions significantly associated with the proteins identified in the cerebellar vermis study. Figure was adapted from Ingenuity® Pathway Analysis software. Significance threshold set at $-\log(0.05)$. 
6.3 Metabolic Pathways

Using the IPA software tool (see Figure 6.3.1 for results), a number of metabolic pathways were found to be associated to the proteins altered in the BA9 white and grey matter and cerebellar vermis in the alcohol groups studied. A significant correlation to the pentose phosphate pathway, glycolysis and gluconeogenesis was seen across the three brain regions in both alcohol groups. This may be related to thiamine deficiency and subsequent energy deprivation as discussed in previous chapters. Inositol metabolism was also significantly related to the vermis, BA9 grey matter and white matter in both alcohol groups. Zhang, et al., detected a significant decrease in inositol metabolism in the cerebral cortex and hippocampus but not the cerebellum in mice that had been fed ethanol chronically (Zhang et al., 1997). Interestingly, using in vivo and ex vivo NMR spectroscopy, a decrease in myo-inositol has been detected in experimental hepatic encephalopathy (Claudia, 2007).

A number of metabolic pathways appeared to be significantly altered in the vermis study suggesting a more complex, multifactorial pathophysiology in this brain region in alcoholics. Interestingly, this included D-glutamine and D-glutamate metabolism. The glutamate-glutamine cycle is the principle means of cerebral ammonia detoxification and is largely localised to astrocytes (Albrecht and Norenberg, 2006). This finding may therefore indicate glia-associated changes related to an increased need for ammonia clearance caused by alcohol-related liver dysfunction. However, the uncomplicated alcoholic group was also linked to this pathway, which does not support this hypothesis. The citrate cycle was also significantly associated to the vermis study. Ammonia-induced inhibition of the citrate cycle has been previously
Figure 6.3.1: Metabolic pathways significantly associated to the proteins identified in the vermis, BA9 grey (GM) and white matter (WM) from uncomplicated and complicated alcoholics (UA; CA respectively). Asterisks (*) mark the presence of only one gene associated to a particular pathway. Figure was adapted from Ingenuity® Pathway Analysis software. Significance threshold set at \(-\log(0.05)\).
described (Felipo and Butterworth, 2002), so this association may also be related to liver dysfunction.

Many of these metabolic pathways are linked via common intermediary metabolites, hence changes in one particular pathway may have consequences for others. For example, changes in fructose-bisphosphate aldolase C activity can affect glyceraldehyde-3P, which is a common substrate of glycolysis, inositol metabolism and the PPP, thereby affecting the metabolic flux through these pathways. The changes we are detecting in these alcoholic brain proteomes are perhaps dampened by this ability to shift and counterbalance metabolic modes due to the stress inflicted by heavy alcohol consumption. This is depicted in the Figures 6.3.2-4 below. These figures appear to become increasingly more complex, moving from the BA9 white matter study, to the BA9 grey matter study and finally the vermis study. This trend may reflect an increase in cell population heterogeneity. Yet, no significant differences were seen in these pathways between the BA9 grey and white matter from healthy individuals (Chapter 3, page 89). Thus, this trend may be indicative of different regional sensitivities to alcohol or other related factors. Also noteworthy in these diagrams are the similarities between the two groups of alcoholics (highlighted in red and orange).
Figure 6.3.2: Metabolic networks associated with the alcohol-related damage to BA9 white matter. All pathways are common to both groups of alcoholics with the exception of one branch from ‘glycolysis/gluconeogenesis’ in orange, which was seen only in uncomplicated alcoholics. ACO2, aconitase 2 mitochondrial; ALDOC, fructose-bisphosphate aldolase C; CKB, creatine kinase brain; DPYSL2, dihydropyrimidinase-like 2; GADPH, glyceraldehyde-3-phosphate dehydrogenase; P4HB, protein disulfide isomerase; PGAM1, phosphoglycerate mutase 1; TALDO, transaldolase; TKT, transketolase. This figure was adapted from Ingenuity® Pathway Analysis software.
Figure 6.3.3: Metabolic pathways associated with the alcohol-related damage to BA9 grey matter. All metabolic pathways are common to both groups of alcoholics with the exception of one branch from ‘glycolysis/gluconeogenesis’: the pathway in red was unique to alcoholics with liver cirrhosis. ACO2, aconitase 2 mitochondrial; ALDH7A1, aldehyde dehydrogenase 7 family, member A1; ALDOC, fructose-bisphosphate aldolase C; CKB, creatine kinase brain; DPYSL2, dihydropyrimidinase-like 2; ENO1, alpha enolase 1; OXCT1, 3-oxoacid CoA transferase 1; P4HB, protein disulfide isomerase; PDHB, pyruvate dehydrogenase beta, PGAM1, phosphoglycerate mutase 1; PKM2, pyruvate kinase; TKT, transketolase. This figure was adapted from Ingenuity® Pathway Analysis software.
Figure 6.3.4: Metabolic pathways associated with the alcohol-related damage to the cerebellar vermis. All metabolic pathways are common to both groups of alcoholics with the exception of two branches from the glycolysis/gluconeogenesis and one branch from the β-alanine metabolic pathways. These exceptions are highlighted in red and depict genes associated with the group of alcoholics with liver cirrhosis. ACO2, aconitase 2 mitochondrial; ALDH1A1, aldehyde dehydrogenase 1 family, member A1; ALDH2, aldehyde dehydrogenase 2 family (mitochondrial); ALDOC, fructose-bisphosphate aldolase C; CKB, creatine kinase brain; CRMP1, collapsin response mediator protein 1; DPYSL2, dihydropyrimidinase-like 2; ENO1, alpha enolase 1; FH, fumarate hydratase; GLUD1, glutamate dehydrogenase 1; LDHB, lactate dehydrogenase B; PDHB, pyruvate dehydrogenase beta, PGAM1, phosphoglycerate mutase 1; PKM2, pyruvate kinase; PRDX6, peroxiredoxin 6; TKT, transketolase; TP11, tiosephosphate isomerase 1. This figure was adapted from Ingenuity® Pathway Analysis software.
6.4 Signaling Pathways

Five signaling pathways were significantly associated with the proteins identified in these studies (see Figure 6.4.1). Significant associations to nuclear factor E2-related factor 2 (NRF-2) mediated oxidative stress response were seen in both alcohol groups in all brain regions studied. In response to oxidative stress, the NRF-2 transcription factor translocates from the cytoplasm into the nucleus and transactivates the expression of genes with antioxidant activity (Ramsey et al., 2007). Recently, it has been suggested that NRF-2 may underlie a feedback system limiting oxidative load during chronic metabolic stress (Shih et al., 2005). As oxidative stress is a salient feature of many neurological diseases including alcoholism, the NRF-2 signaling pathway is an attractive therapeutic target (van Muiswinkel and Kuiperij, 2005).

Figure 6.4.1: Signaling pathways significantly associated to the proteins identified in the vermis, BA9 grey (GM) and white matter (WM) from uncomplicated and complicated alcoholics (UA; CA respectively). This figure was adapted from Ingenuity® Pathway Analysis software. Significance threshold set at −log(0.05).
Axonal guidance signaling was significantly associated to the BA9 region studies. Ethanol exposure has been proposed to disrupt the way axons respond to guidance cues and in recent experiments was shown to disrupt growth-cone motility associated with axonal guidance in cultured hippocampal neurons (Lindsley et al., 2006). Why this association was not seen in the cerebellar vermis study is unclear. Other significant associations included GABA receptor signaling in both alcohol groups in the BA9 grey matter study and vascular endothelial growth factor (VEGF) signaling in the vermis of alcoholics with liver cirrhosis. Ethanol is known to be a potent modulator of GABAergic neurotransmission (Brailovsky and Garcia, 1999), and a number of studies have shown differences in the relative expression of several GABA<sub>A</sub> receptor subunits in the superior frontal cortex of human chronic alcoholics (Lewohl et al., 1997; Lewohl et al., 2001b; Mitsuyama et al., 1998). The significant association to GABA receptor signaling in this same brain region of alcoholics (BA9 grey matter) appears to correlate with these previously reported changes (Lewohl et al., 1997; Lewohl et al., 2001b; Mitsuyama et al., 1998). VEGF is an important signaling protein in blood vessel growth. Recent data has indicated that ethanol increases the production of VEGF mRNA and protein in cell cultures in a dose-related fashion (Gu et al., 2005). Again, why this association was unique to the vermis of alcoholics with liver cirrhosis is unknown. The relationships between these signaling pathways and the genes involved are depicted below (See Figures 6.4.2-3).
Figure 6.4.2: Signaling pathways associated with the BA9 grey and white matter studies. No difference was seen between the two alcohol groups in both studies. Genes and pathways in black are common to both regions (grey and white), whereas those highlighted in red are unique to the BA9 grey matter study. CLTB, clathrin light; CTSD, cathepsin D; DNM1, dynamin 1; DPYS12, dihydropyrimidinase-like 2; FTL, ferritin light chain; GNAO1, guanine nucleotide binding protein alpha; GNB1, guanine nucleotide binding protein beta 1; GNB2, guanine nucleotide binding protein beta 2; HSPA2, heat shock 70kDa protein 2; HSPA8, heat shock 70kDa protein 8; NAPB, N-ethylmaleimide-sensitive factor attachment protein, beta; NSF, N-ethylmaleimide-sensitive factor; VCP, valosin-containing protein.
Figure 6.4.3: Signaling pathways associated with the cerebellar vermis study. Genes and pathways in black are common to both groups of alcoholics, whereas those highlighted in red are unique to the alcoholics with liver cirrhosis. ACTB, actin beta; ACTG1, actin gamma 1; DNM1, dynamin 1; DPYSL2, dihydropyrimidinase-like 2; GRB2, growth factor receptor-bound protein 2; HSPA9, heat shock 70kDa protein 9B; VCP, valosin-containing protein.
6.5 Biomarker Comparison Analyses

Using IPA-Biomarker™, filters were applied to the datasets to identify and prioritize the most relevant and promising molecular biomarker candidates. The biomarker filter was set based on the following contextual information;

a) Genes expressed in humans,
b) Genes expressed in tissues restricted to the nervous system, and
c) Genes that are mechanistically connected to metabolic disease, neurological disease, nutritional disease, organismal injury/abnormalities and/or psychological disorders.

Using these filters, biomarker candidates that discriminate between or are common to the different groups of alcoholics and brain regions were isolated. No biomarkers were identified as unique to either the uncomplicated alcoholics or alcoholics with liver cirrhosis across all the brain regions studied. However, two markers were common to both alcohol groups across all regions: transketolase and phosphatidylethanolamine binding protein 1 (PEBP1). Transketolase, a thiamine-dependent enzyme, was shown to have reduced activity in autopsy samples of vermis from alcoholic patients with WKS (Butterworth et al., 1993). These authors also showed reduced transketolase activity in the cerebellum and prefrontal cortex of alcoholic patients with liver cirrhosis but without WKS (Lavoie and Butterworth, 1995). The studies described in this thesis indicated a marked decrease in transketolase levels not only in cirrhosis-complicated alcoholics, but also in the brains of ‘neurologically uncomplicated’ alcoholics. This suggests that to some degree, all alcoholics may be thiamine deficient and the diagnostic criteria for WKS are not
stringent enough to pick up sub-clinical thiamine deficiencies or early stages of this syndrome.

In neural tissue, PEBP was originally localized to oligodendrocytes (Moore et al. 1996; Roussel et al., 1988) and Schwann cells (Moore et al., 1996). Phosphatidylethanolamine (PE) is the major phospholipid component of myelin sheath elaborated by such cells and authors initially postulated a role for PEBP in membrane biogenesis and maintenance of membranes (Frayne et al., 1999). Carrasco and colleagues studied phospholipid biosynthesis in hepatocytes isolated from rats fed ethanol chronically and demonstrated that ethanol induced specific effects on the biosynthesis of PE (Carrasco et al., 1996). Interestingly, following western blot and RT-PCR analyses in mouse brain, liver and plasma, PEBP1 was recently suggested as a potential plasma biomarker for acute liver failure and an important protein in the pathogenesis of this acute disorder (Lv et al., 2007).

Yet, further studies on rat brain have suggested a functional role beyond that of the organization of the myelin sheath (Frayne et al., 1999), including cholinergic neuronal stimulatory activity (Butterfield et al., 2006). A recent proteomics study also using 2D-GE found changes in PEBP in the nucleus accumbens of alcohol-preferring rats (Witzmann et al., 2003). Interestingly, the cholinergic interneurons of the nucleus accumbens have been reported to play a pivotal role in substance abuse, including acute ethanol exposure (Herring et al., 2004). In the studies described in this thesis, PEBP was identified in both uncomplicated alcoholics and alcoholics with liver cirrhosis in the prefrontal region and in the vermis. The precise pathophysiologic
mechanism of PEBP involvement in alcohol-related brain damage is unclear and further studies on the role of this protein may reveal some interesting insights.

In Alzheimer’s disease, cognitive decline is suggested to be primarily due to a cholinergic impairment (Butterfield et al., 2006). Perhaps it is therefore not surprising that PEBP has been identified as a specifically oxidized protein in Alzheimer's disease brains (Castegna et al., 2003). Indeed, five protein spots isolated in the cerebellar vermis of alcoholics with liver cirrhosis were identified as PEBP, as opposed to one PEBP protein spot in all other groups/regions (See Table 5.4.1, page 162). Whether these PEBP protein spots are post-translational modifications, such as oxidation, and why these spots occurred only in the vermis of alcoholics with liver disease also warrants further study.

The same filters were applied to identify regional biomarkers. Two genes were uniquely associated to the BA9 white matter study, aminolevulinate synthase, annexin A2\(^1\). Another two genes, clathrin light chain and capthesin D were associated to the BA9 grey matter study\(^1\) and lastly, actin gamma and aldehyde dehydrogenase 1 and 2 were unique to the vermis study\(^2\). Further study into the regional alterations of these genes may help elucidate why chronic alcohol consumption affects the brain in a region-specific manner.

\(^1\) Please refer to Chapter 4 for information of the proteins associated with these genes (page 110)

\(^2\) Please refer to Chapter 5 for discussion of the proteins associated with these genes (page 149)